DOCKET NO.: 0163-0758-0

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: :GROUP ART UNIT: 1655

SHINYA KURATA ET AL: :

SERIAL NO.: 09/556,127 :EXAMINER: FREDMAN

FILED: APRIL 20, 2000

FOR: METHOD FOR DETERMINING A CONCENTRATION OF TARGET NUCLEIC ACID MOLECULES, NUCLEIC ACID PROBES FOR THE METHOD, AND METHOD FOR ANALYZING DATA OBTAINED BY THE METHOD

DECLARATION UNDER 37 C.F. R. §1.132

HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS

WASHINGTON, D.C. 20231

SIR:

Now comes Shinya KURATA who deposes and states:

1. That I am a graduate of Shimane University at Matsue city and received my Master degree in the year 1993.

2. That I have been employed by Kankyo Engineering Co., Ltd. for 10 years as a researcher in a field of biotechnology.

3. I am an inventor of 09/556,127 and am familiar with the prosecution history thereof.

4. The following experiments were performed by me or under my direct supervision

and control.

5. The following experiments demonstrate that all the probes labeled with the fluorescent dyes listed in the claims yield consistent quenching rates when used in a hybridization experiments. The experiments described herein were performed as disclosed in the present application.

6. The probe labeled with BODIPY FL was prepared as follows:

The phosphate group of the deoxycytidylic acid was modified with an amino-linker using a 5' Amino-Modifier C6 kit (Glen Research Inc., USA). Deoxyriboolygonucleotides with base sequences described in the foot-note of the following Table 1 were synthesized using this modified deoxyribocytidylic acid and other deoxyribonucleosides by using a DNA synthesizer, ABI 394™ (Perkin-Elmer Corp.). After the synthesis, protecting groups were released using a 28% ammonia solution. The product was dried and dissolved in a buffer solution (pH, 9.0) of 0.5M $NaHCO_3/Na_2CO_3$ The synthesized deoxyriboolygonucleotides were purified using a NAP-10 column (a product of Pharmacia Inc , Sweden).

A FluoroReporter Kit F-6082 (Molecular Probes, Inc.), which contains BODIPY FL propionic acid succinimidilyl ester and reagents for conjugating the compound to the amine derivative of the above synthesized deoxyriboolygonucleotides, was employed to label the above synthesized deoxyriboolygonucleotides with BODIPY FL at 5'-phosphate. The BODIPY FL-labeled deoxyriboolygonucleotides were purified using the above column and a reverse High Performance Liquid Chromatographic method with a SEP-PAK C18 column (6× 250 mm), an elution solvent (0.5N TEAA 5%CH_3CN) and a gradient solvent B (0.5N TEAA 40%CH_3CN).

7. The probes labeled with the other fluorescent dyes than BODIPY FL were prepared as described above for the BODIPY FL-labeled probes and deoxyriboolygonucleotides with base sequences described in the foot-note of the following

tables 2 and 3 were used in the experiments of each table.

8. The target nucleic acids with base sequences described in the foot-notes of the tables were synthesized by using the DNA synthesizer, which nucleic acids were capable of hybridizing with the above corresponding probes and had the same chain lengths as those of the probes. The target nucleic acids were not labeled.

9. The aforementioned probes were hybridized to the target nucleic acids as follows:

10. The hybridization was performed at 51 ℃ and the hybridization mixture contained:

Target nucleic acid	320 nM (final concentration)
Nucleic acid probe	80 nM (final concentration)
NaCl	50 nM (final concentration)
$MgCl_2$	1 nM (final concentration)
Tris-HCl buffer (pH 7.2)	100 nM (final concentration)
"MiliQ" purified water	1.6992 mL
final whole volume	2.0000 mL

11. The hybrids formed were measured with excited light at the optimal wave lengths as described in the tables. The fluorescent colors were also measured at the optimal wave lengths as described in the tables.

12. The results of the above-hybridizations yielded the following rates of decreased fluorescent intensity (quenching rates):

Table 1. Experimental data for the hybridization results

Fluorescent dyes	Excitation (nm)	Emission (nm)	Decrease in fluorescent intensity (%) Experimental No.					
			1	2	3	4	5	the mean
BODIPY FL	500	520	91	93	93	92	92	92
BODIPY FL/CL3	500	520	94	96	95	95	95	95
6-joe	515	550	66	67	67	67	66	67
TMR	545	580	90	89	89	88	90	89
Alexa 488	480	520	55	56	53	54	54	54
Alexa 532	515	545	38	40	39	41	39	39

Sequences for Table 1.

Probe: 5' CCCCCCCCCC CCTTTTT 3'

Target nucleic acid: 5' AAAAAGGGGG GGGGGGG 3'

Table 2. Experimental data for the hybridization results

Fluorescent dyes	Excitation (nm)	Emission (nm)	Decrease in fluorescent intensity (%) Experimental No.					
			1	2	3	4	5	the mean
BODIPY FL	500	520	85	84	85	86	85	85
BODIPY FL/CL3	500	520	89	90	88	91	90	90
6-joe	515	550	65	63	64	64	63	64
TMR	545	580	82	81	83	81	81	82
Alexa 488	480	520	50	50	49	50	51	50
Alexa 532	515	545	32	33	32	33	31	32

Sequences for Table 2

Probe:5' CCTTCCCACA TCGTTT 3'

Target nucleic acid: 5' AAACGATGTG GGAAGG 3'

Table 3. Experimental data for the hybridization results

Fluorescent dyes	Excitation (nm)	Emission (nm)	Decrease in fluorescent intensity (%) Experimental No.					
			1	2	3	4	5	the mean
BODIPY FL	500	520	81	80	82	82	79	81
BODIPY FL/CL3	500	520	82	84	84	82	83	83
6-joe	515	550	59	62	60	60	62	61
TMR	545	580	79	78	79	77	78	78
Alexa 488	480	520	47	47	48	47	47	47
Alexa 532	515	545	30	31	33	34	32	32

Sequences for Table 2

Probe: 5' CGGAAAATAG ACCAATAGGC AG 3'

Target nucleic acid: 5' CTGCCTATTG GTCTATTTTC CG 3'

The rate of fluorescent intensity was measured using the following formula:

Rate of decreased fluorescent intensity = 100 - {(fluorescent intensity after hybridization)/(fluorescent intensity before hybridization (before addition of a target nucleic acid in the reaction mixture))} × 100 .

13. The results of these experiments show that all the probes labeled with the fluorescent dyes listed in the claims yield consistent quenching rates and almost equivalent rates even when those probes are applied to different base sequences.

14. The undersigned petitioner declares further that all statements made herein of his

own knowledge are true and that all statements made on information are believed to be true. Further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

15. Further deponent saith not.

Shinya Kurata

Signature Shinya KURATA

January 22, 2002

Date January 22, 2002